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**Scientific Opinion on application (EFSA-GMO-UK-2006-34) for the placing
on the market of genetically modified maize 3272 with a thermotolerant
alpha-amylase, for food and feed uses, import and processing under
Regulation (EC) No 1829/2003 from Syngenta Crop Protection AG**

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Abstract: Maize 3272 contains a single insert consisting of the amy797E and the pmi cassettes, expressing a thermotolerant alpha-amylase (AMY797E) and a phosphamannose isomerase (PMI). Bioinformatic analyses and genetic stability studies did not raise safety issues. The levels of the AMY797E and PMI proteins in maize 3272 have been sufficiently analysed. In the absence of an appropriately performed comparative assessment, the EFSA Panel on Genetically Modified Organisms (GMO) was not in the position to conclude either on the compositional, agronomic and phenotypic characteristics of maize 3272 or on its nutritional assessment, on the basis of the data provided. The safety assessment could therefore not be completed, and has focused mainly on the newly expressed proteins. No indications of safety concern over the toxicity of the AMY797E and PMI proteins and over the allergenicity of the PMI protein were identified. The Panel could not conclude on the potential for de novo allergic sensitisation of the AMY797E protein. The Panel has identified a gap in the data on the agronomic and phenotypic characterisation of GM maize 3272 and considers that uncertainty over these characteristics remains. However, considering the scope of this application, a weight of evidence approach from different sources of available data and the poor ability of maize to survive outside cultivated land, the Panel concluded that there is very little likelihood of any adverse environmental impacts due to the accidental release into the environment of viable grains from maize 3272. Considering its intended uses as food and feed, interactions with the biotic and abiotic environment were not considered to be an issue. Risks associated with a theoretically possible horizontal gene transfer from maize 3272 to prokaryotes have been analysed and did not raise safety concerns. The monitoring plan and reporting intervals were in line with the intended uses of maize 3272

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SCIENTIFIC OPINION

Scientific Opinion on application (EFSA-GMO-UK-2006-34) for the placing on the market of genetically modified maize 3272 with a thermotolerant alpha-amylase, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta Crop Protection AG¹

EFSA Panel on Genetically Modified Organisms (GMO)^{2,3}

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published on 21 August 2013, replaces the earlier versions published on 20 and 21 June 2013*

ABSTRACT

Maize 3272 contains a single insert consisting of the *amy797E* and the *pmi* cassettes, expressing a thermotolerant alpha-amylase (AMY797E) and a phosphamannose isomerase (PMI). Bioinformatic analyses and genetic stability studies did not raise safety issues. The levels of the AMY797E and PMI proteins in maize 3272 have been sufficiently analysed. In the absence of an appropriately performed comparative assessment, the EFSA Panel on Genetically Modified Organisms (GMO) was not in the position to conclude either on the compositional, agronomic and phenotypic characteristics of maize 3272 or on its nutritional assessment, on the basis of the data provided. The safety assessment could therefore not be completed, and has focused mainly on the newly expressed proteins. No indications of safety concern over the toxicity of the AMY797E and PMI proteins and over the allergenicity of the PMI protein were identified. The Panel could not conclude on the potential for *de novo* allergic sensitisation of the AMY797E protein. The Panel has identified a gap in the data on the agronomic and phenotypic characterisation of GM maize 3272 and considers that uncertainty over these characteristics remains. However, considering the scope of this application, a weight of evidence approach from different sources of available data and the poor ability of maize to survive outside cultivated land, the Panel concluded that there is very little likelihood of any adverse environmental impacts due to the accidental release into the environment of viable grains from maize 3272. Considering its intended uses as food and feed, interactions with the biotic and abiotic environment were not considered to be an issue. Risks associated with a theoretically possible horizontal gene transfer from maize 3272 to prokaryotes have been analysed and did not raise safety concerns. The monitoring plan and reporting intervals were in line with the intended uses of maize 3272.

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¹ On request from the Competent Authority of the United Kingdom for application (EFSA-GMO-UK-2006-34) submitted by Syngenta Crop Protection AG, Question No EFSA-Q-2006-026, adopted on 30 May 2013.

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KEY WORDS

GMO, 3272 maize, food and feed safety, environment, import and processing, Regulation (EC) No 1829/2003, thermotolerant alpha-amylase.

SUMMARY

Following the submission of an application (Reference EFSA-GMO-UK-2006-34) under Regulation (EC) No 1829/2003 from Syngenta Crop Protection AG, the Panel on Genetically Modified Organisms of the European Food Safety Authority (EFSA GMO Panel) was asked to deliver a scientific opinion on the safety of genetically modified (GM) maize 3272 (Unique Identifier SYN-E3272-5) for import and processing and for food and feed uses. Maize 3272 was developed to express a chimeric thermotolerant alpha-amylase (AMY797E) and a phosphomannose isomerase (PMI), as a selectable marker.

In delivering its scientific opinion, the EFSA GMO Panel considered the application EFSA-GMO-UK-2006-34, additional information provided by the applicant (Syngenta Crop Protection AG) and the scientific comments submitted by the Member States. The scope of application EFSA-GMO-UK-2006-34 is for food and feed uses and import and processing of maize 3272 and all derived products, but excludes cultivation in the European Union (EU).

The EFSA GMO Panel evaluated maize 3272 with reference to the intended uses and appropriate principles described in the EFSA GMO Panel guidance documents for the risk assessment of GM plants and derived food and feed. The scientific risk assessment evaluation included molecular characterisation of the inserted DNA and expression of target proteins. A comparative analysis of agronomic traits and composition was undertaken, and the safety of the new proteins, as individual proteins and in combination, the changed levels of natural constituents and the whole food/feed were evaluated with respect to potential toxicity, allergenicity and nutritional quality. An evaluation of environmental impacts and the post-market environmental monitoring plan were undertaken.

Maize 3272 has been genetically modified to express the AMY797E and PMI proteins. The AMY797E protein is a chimeric thermotolerant alpha-amylase encoded by gene segments derived from three parental alpha-amylase genes originating from strains of the archeal order *Thermococcales*. The PMI protein is a phosphomannose isomerase encoded by the *pmi* gene (also known as *manA*) derived from *Escherichia coli*. Expression of PMI enables transformed maize cells to utilise mannose and therefore to survive on media in which mannose is the sole source of carbon.

The molecular characterisation data established that the GM maize 3272 contains a single insert consisting of the *amy797E* and the *pmi* cassettes. Bioinformatic analyses and genetic stability studies did not raise safety issues. The levels of the AMY797E and PMI protein in maize 3272 have been sufficiently analysed.

The EFSA GMO Panel could not conclude on the comparative assessment of the compositional, agronomic and phenotypic characteristics of maize 3272, on the basis of the data provided. In the absence of an appropriately performed comparative assessment, the safety assessment could not be completed and has focused mainly on the newly expressed proteins AMY797E and PMI.

The AMY797E and PMI proteins did not show significant similarity to known toxins in bioinformatic analyses. The EFSA GMO Panel concluded that administration of the AMY797E protein to rats for 28 days did not induce adverse effects up to the highest dose tested. Based on all the available information, the EFSA GMO Panel considers that there are no indications that the newly expressed PMI protein in maize 3272 may be allergenic. In relation to the AMY797E protein, the EFSA GMO Panel could not conclude on the *de novo* sensitisation potential of the protein.

The EFSA GMO Panel considers that both the repeated-dose 90-day oral toxicity study and the feeding study in broiler chickens, in which material derived from a negative segregant is administered as the control material, are not adequate for the safety assessment of food/feed from GM plants. Therefore, the Panel did not consider these studies in its evaluation of maize 3272.

The application EFSA-GMO-UK-2006-34 concerns food and feed uses, import and processing. Therefore, there is no requirement for scientific information on possible environmental effects

associated with the cultivation of maize 3272. The EFSA GMO Panel has identified a gap in the data on the agronomic and phenotypic characterisation of GM maize 3272 and considers that uncertainty over these characteristics remains. However, considering the scope of this application, a weight of evidence approach from different sources of available data and the poor ability of maize to survive outside cultivated land, the EFSA GMO Panel concluded that there is very little likelihood of any adverse environmental impacts as a result of the accidental release into the environment of viable grains from maize 3272. In the case of accidental release into the environment of viable grains of maize 3272, there are no indications of an increased likelihood of spread and establishment of feral maize 3272 plants. Considering its intended uses as food and feed, interactions with the biotic and abiotic environment were not considered to be an issue. Risks associated with a theoretically possible horizontal gene transfer from maize 3272 to prokaryotes (i.e. bacteria, *Archaea*) have been analysed and did not raise safety concerns. The post-market environmental monitoring plan and reporting intervals were in line with the intended uses of maize 3272.

In the absence of an appropriately performed comparative assessment by the applicant, the EFSA GMO Panel was not in the position to complete its risk assessment on maize 3272 and therefore does not conclude on the safety of maize 3272 compared with its conventional counterpart with respect to potential effects on human and animal health. However, the EFSA GMO Panel concluded that maize event 3272 is unlikely to have any adverse effect on the environment in the context of its intended uses.

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BACKGROUND

On 6 March 2006, EFSA received from the Competent Authority of the United Kingdom an application (Reference EFSA-GMO-UK-2006-34), for authorisation of genetically modified maize 3272 (Unique Identifier SYN-E3272), submitted by Syngenta Crop Protection AG within the framework of Regulation (EC) No 1829/2003 on genetically modified food and feed (EC, 2003) for food and feed uses, import and processing.

After receiving the application EFSA-GMO-UK-2006-34 and in accordance with Articles 5(2)(b) and 17(2)(b) of Regulation (EC) No 1829/2003, EFSA informed the Member States as well as the European Commission and made the summary of the dossier publicly available on the EFSA website. EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of Regulation (EC) No 1829/2003. On 4 June 2007, the applicant provided EFSA with additional information requested under completeness check (requested on 26 March 2007) and on 6 July 2007 EFSA declared the application as valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

EFSA made the valid application available to Member States and the European Commission and consulted nominated risk assessment bodies of the Member States, including the national Competent Authorities within the meaning of Regulation (EC) No 1829/2003 and Directive 2001/18/EC (EC, 2001) following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1829/2003, to request their scientific opinion. The Member State bodies had three months after the date of receipt of the valid application (until 6 October 2007) within which to make their opinion known.

The GMO Panel carried out a scientific assessment of genetically modified (GM) maize 3272 taking into account the appropriate principles described in the Guidance Document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed (EFSA, 2006).

On 23/11/2007, 14/04/2008, and on 06/05/2010 the GMO Panel asked for additional data on maize 3272 (application EFSA-GMO-UK-2006-34). The applicant provided the requested information on 07/01/2010 and on 01/10/2012. After receipt and assessment of the data package, the GMO Panel finalised its risk assessment of maize 3272.

On 11/07/2012 EFSA informed the applicant that, given that application EFSA-GMO-UK-2006-34 had been processed for six years since its reception and the datasets received did not allow EFSA to conclude on the safety of maize 3272, after 30 September 2012, which corresponded to the latest deadline to deliver additional information as specified by the applicant, EFSA would proceed with the finalisation of the assessment of EFSA-GMO-UK-2006-34 and deliver its opinion based on the information available at that time.

The GMO Panel carried out a scientific assessment of the GM maize 3272 for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003, taking into consideration the scientific comments of the Member States and the additional information provided by the applicant.

In giving its opinion on GM maize 3272 to the European Commission, the Member States and the applicant, and in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003, EFSA has endeavoured to respect a time limit of six months from the receipt of the valid application. As additional information was requested by the EFSA GMO Panel, the time limit of 6 months was extended accordingly, in line with Articles 6(1), 6(2), 18(1), and 18(2) of Regulation (EC) No 1829/2003.

According to Regulation (EC) No 1829/2003, the EFSA opinion shall include a report describing the assessment of the food and feed and stating the reasons for its opinion and the information on which its opinion is based. This document is to be seen as the report requested under Articles 6(6) and 18(6)

of that Regulation, and thus will be part of the overall opinion in accordance with Articles 6(5) and 18(5).

TERMS OF REFERENCE

The GMO Panel was requested to carry out a scientific assessment of the genetically modified maize 3272 for food and feed uses and import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003. Where applicable, any conditions or restrictions which should be imposed on the placing on the market and/or specific conditions or restrictions for use and handling, including post-market monitoring requirements based on the outcome of the risk assessment and, in the case of GMOs or food/feed containing or consisting of GMOs, conditions for the protection of particular ecosystems/environments and/or geographical areas should be indicated in accordance with Articles 6(5)(e) and 18(5)(e) of Regulation (EC) No 1829/2003.

The EFSA GMO Panel was not requested to give an opinion on information required under Annex II to the Cartagena Protocol, nor on the proposals for labelling and methods of detection (including sampling and the identification of the specific transformation event in the food/feed and/or food/feed produced from it), which are matters related to GMO risk management.

ASSESSMENT

1. Introduction

Maize 3272 was developed for use in the dry-grind fuel ethanol process in which the starch contained in cereal grains is hydrolysed into glucose, which is subsequently converted to ethanol by fermentation. In a modification to the existing process, grain from maize 3272 will be mixed with grain from conventional maize varieties, and thus will serve as source of thermotolerant alpha-amylase, which would eliminate the need to add microbially produced enzyme. After the action of the thermotolerant alpha-amylase, the resulting dextrins, maltose and glucose are treated with glucoamylase to completely hydrolyse the dextrins into glucose (saccharification), which is used as the substrate for yeast (*Saccharomyces cerevisiae*) fermentation to produce ethanol.

Maize 3272 expresses a thermotolerant alpha-amylase encoded by the *amy797E* gene, which is composed of DNA sequences from alpha-amylase genes from three thermophilic microorganisms of the order *Thermococcales* (class *Thermococci*; phylum *Euryarchaeota*; domain *Archaea*). Alpha-amylases catalyse the hydrolysis of starch (amylose and amylopectin) by cleavage of alpha-1,4 glucosidic bonds resulting in the production of dextrins, maltose and glucose. Alpha-amylase enzymes of fungal and bacterial origin have been used traditionally in starch processing.

Maize 3272 also expresses a phosphomannose isomerase (PMI) protein as a marker. Expression of PMI enables transformed maize cells to utilise mannose and therefore to survive on media in which mannose is the sole source of carbon.

The scope of application EFSA-GMO-UK-2006-34 is for food and feed uses and import and processing of maize 3272 and all derived products (e.g. starch, syrups, ethanol, maize oil, flakes, coarse and regular grits, coarse and dusted meal, flour, maize germ meal, maize feed, condensed steep water and maize meal).

Event 3272 maize is intended to be cultivated and used in the dry-grind fuel ethanol process outside the EU. The grain is not intended to be used either in other processing applications (e.g. wet milling and dry milling processes) or as a commodity crop. However, it cannot be excluded that the crop originally intended for industrial use could inadvertently enter the food and feed chain, albeit at low levels. In addition, by-products of the dry-grind ethanol process produced from maize and other cereal are widely used as feed (e.g. distillers' dried grains with solubles).

The genetically modified (GM) maize 3272 (Unique Identifier SYN-E3272-5) was assessed with reference to its intended uses, taking account of the appropriate principles described in the Guidance Document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of GM plants and derived food and feed (EFSA, 2006).

The risk assessment presented here is based on the information provided in the application EFSA-GMO-UK-2006-34 submitted in the EU including the additional information from the applicant and the scientific comments that were raised by Member States on this application.

2. Issues raised by Member States

The scientific comments raised by Member States are addressed in Annex G of the EFSA overall opinion and have been considered throughout this EFSA GMO Panel scientific opinion.

3. Molecular characterisation

3.1. Evaluation of relevant scientific data

3.1.1. Transformation process and vector constructs

Maize 3272 was developed by *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*)-mediated transformation of immature maize embryos with strain LBA4404 containing the plasmid vector pNOV7013. The regeneration of the transformed tissue was achieved after a callus phase.⁴ The plasmid vector pNOV7013 included one T-DNA which contained two expression cassettes between the right and left borders:⁵

i) *amy797E* gene cassette

A chimeric thermotolerant alpha-amylase (*amy797E*) gene is under the control of the promoter from the gene encoding the 27 kDa storage protein gamma-zein (GZein) from *Zea mays* and the terminator sequence from the 35S RNA of the *Cauliflower mosaic virus*. The terminator sequence is preceded by a 108 bp fragment comprising the maize phosphoenolpyruvate carboxylase gene intron 9 (PEPC9).

AMY797E is a chimeric protein coded by gene segments derived from three parental alpha-amylase genes; BD5031, BD5064 and BD5063 originating from strains of the archeal order *Thermococcales*. The first two genes were obtained from microbial DNA libraries constructed from pure cultures of *Thermococcus* strains isolated from samples taken from shallow marine hydrothermal systems at 95 °C, pH 7.0 and 85 °C, pH 6.0, respectively. The third gene originated from a microbial DNA library constructed from a primary enrichment culture containing an undetermined number of high-temperature organisms isolated from deep-sea Pacific Ocean with prevailing temperatures of 90 °C. Based on sequence comparisons, the most likely source of BD5063 is either a *Pyrococcus* or *Thermococcus* species. These three alpha-amylase enzymes were chosen for their superior activity under high-temperature, low-Ca²⁺ or low-pH conditions, all relevant to the starch liquefaction step of maize processing. The chimeric *amy797E* gene was assembled from the parental sequences and its sequence has been codon-adapted to achieve a high level of expression in maize. The final AMY797E protein intended to be expressed in event 3272 maize is 460 amino acids long and carries the maize gamma-zein signal sequence as an N-terminal fusion and an endoplasmic reticulum (ER) retention signal as a C-terminal fusion.

ii) *pmi* gene cassette (used as selectable marker)

The *pmi* gene (also known as *manA*) is derived from *Escherichia coli* and encodes a phosphomannose isomerase (PMI) enzyme. The gene is under the control of the promoter and first intron region of the *Zea mays* polyubiquitin gene and the *nos* terminator of *A. tumefaciens*. Expression of PMI enables transformed maize cells to utilise mannose, and therefore to survive on media in which mannose is the sole source of carbon.

3.1.2. Transgene constructs in the GM plant⁶

Southern analyses were used to determine the structure and copy number of insert(s) in maize 3272 genomic DNA. Southern analyses demonstrated that maize 3272 contains a single insert and single copies of the *amy797E* gene, the *pmi* gene, the maize polyubiquitin promoter plus intron (UbiInt) and the gamma zein promoter region (GZein) derived from plasmid pNOV7013. No vector backbone sequences were detected with the probe covering the entire vector backbone.⁷

⁴ Technical Dossier/Appendix 1.

⁵ Technical Dossier/Sections C1–C3.

⁶ Technical Dossier/Section D2.

⁷ Additional information Jan 2010/Appendix 1.

The nucleotide sequences of the insert as well as both 5' and 3' flanking regions were determined and indicated that the sequence of the T-DNA insert in maize 3272 was preserved except for the deletion of 23 bp of the 5' right border and 7 bp of the 3' left border. These deletions did not affect the functionality of the T-DNA insert. In addition, the insert contains the following sequences derived from the Ti plasmid of *A. tumefaciens*: (1) 143 bp of the right border region including 101 bp of the promoter region of the *nos* gene at the 5' end of the insert and (2) 53 bp of the left border region at the 3' end of the insert. This is of relevance for horizontal gene transfer (see section 6.1.1.2) taking into consideration that the insert also contains 253 bp of the terminator region of the *nos* gene at the 3' end of the insert.

An updated (2012) bioinformatic analysis of the genomic sequences of the pre-insertion site and of the sequences flanking the insert in maize 3272 was carried out to assess any potential interruption of known maize genes. BLASTN searches were performed against plant EST (Expressed Sequence Tag) and non-redundant nucleotide databases and BLASTX searches against a non-redundant amino acid database. The analysis showed that the 3' region of the 3272 insert is highly repetitive and shares strong similarity with a transposon sequence. This region is located 500 bp downstream of the genome-to-insert junction. The genomic sequence flanking the 3' region of the 3272 insert had alignments to three proteins, including one uncharacterised protein from maize, one predicted protein from *Bos taurus* (cow), and one vacuolar pyrophosphatase from *Zygophyllum xanthoxylum*. All alignments were to short sequences not immediately adjacent to the junction. The 5' flanking sequence did not align to any of the proteins mentioned above. The results did not indicate the interruption of any known endogenous gene in maize 3272. The results also confirmed that the insert is located in the nuclear genome.⁸

The applicant provided (2012) a BLASTX analysis of the entire T-DNA insert and its junctions. Using the FARPP database and the *Codex Alimentarius* and EFSA recommendations (EFSA, 2010a; 2011a) regarding the threshold for potential allergenicity, a match of eight identical amino acids occurred between the PMI sequence and α -parvalbumin from *Rana* species CH2001. The PMI expression unit is identical to the one assessed in maize event MIR162 and this match has been reported and evaluated previously (EFSA, 2012). Another single match of eight identical amino acids was identified between the sequence encoding AMY797E and two known allergens, Per a 3.01 and Per a 3 from *Periplaneta americana* (American cockroach). The relevance of these similarities is evaluated in section 5.1.3. Bioinformatic analysis revealed no relevant similarities to known toxic proteins.⁹

3.1.3. Information on the expression of the insert

The levels of the AMY797E and PMI proteins were determined by enzyme-linked immunosorbent assay (ELISA) in two maize hybrids containing the single event 3272 using samples from a field trial at Bloomington, Illinois, USA (2003).¹⁰ Considering the scope of the application, the AMY797E and PMI protein levels in grain are considered the most relevant. The mean AMY797E level for grain was 1 259 $\mu\text{g/g}$ dry weight (dw) (range 908–1 562 $\mu\text{g/g}$ dw) for hybrid A and 1 335 $\mu\text{g/g}$ dw (range 893–1 730 $\mu\text{g/g}$ dw) for hybrid B. The mean PMI level for grain was <0.5 $\mu\text{g/g}$ dw (expressed as the average of quantifiable values; range <LOQ¹¹ to 0.7 $\mu\text{g/g}$ dw) for hybrid A and 0.7 $\mu\text{g/g}$ dw (range 0.5–0.9 $\mu\text{g/g}$ dw) for hybrid B.

In another trial in Bloomington (2007), the levels of the AMY797E and PMI proteins were also determined in two maize hybrids, one containing only the 3272 event the other also containing Bt11, MIR604 and GA21 as stacked events. The mean AMY797E level for grain in the single event 3272 hybrid was 1 493 $\mu\text{g/g}$ dw (range 1 111–1 991 $\mu\text{g/g}$ dw) and for event 3272 in the stack was 1 322 $\mu\text{g/g}$ dw (range 997–1 736 $\mu\text{g/g}$ dw). The mean PMI level for grain of the single event 3272 was 1.93 $\mu\text{g/g}$ dw (range 1.43–2.34 $\mu\text{g/g}$ dw) and for event 3272 in the stack was 4.17 $\mu\text{g/g}$ dw (range

⁸ Additional information Oct 2012/Appendices A-1_02 –A-1_03.

⁹ Additional information Oct 2012/Appendices A-1_04 –A-1_09.

¹⁰ Confidential information/Appendix 5.

¹¹ LOQ for PMI in kernels (maturity) is 0.33 $\mu\text{g/g}$ dw (Confidential information/Appendix 5).

3.01–8.13 µg/g dw). Higher levels of PMI recorded for the stack with several events reflect the fact that PMI is also expressed from the MIR604 event present within this stack.¹² Variations in protein expression values are not unexpected and can be due to differences in genetic background of the plants and/or environmental variables. The safety of the PMI protein is evaluated in section 5.1.2.

3.1.4. Inheritance and stability of inserted DNA

Genetic stability of the inserted DNA was studied over multiple generations (backcross populations BC1 to BC4 with maize inbred line NP2222 as the recurrent parent) of maize 3272 by the polymerase chain reaction (PCR) and Southern analyses. Individual plants were assayed for the presence of the *amy797E* gene by PCR and the expected Mendelian inheritance ratio of positive and negative plants was demonstrated. The restriction enzyme/probe combination used in the Southern analysis was sufficient to conclude that the generations tested retained the single copy insert.¹³ Analysis of the expression of AMY797E and PMI proteins over multiple (four backcross) generations indicated phenotypic stability of the traits in maize 3272.¹⁴

The inheritance pattern of the trait was consistent with a single genetic locus segregating in a Mendelian fashion.

In conclusion, the stability of the inserted DNA and associated traits was confirmed over several generations.

3.2. Conclusion

The molecular characterisation data provided by the applicant established that the GM maize 3272 contains one copy of the T-DNA consisting of the *amy797E* and the *pmi* cassettes. No other parts of the initial plasmid used to obtain the DNA fragment for transformation were detected in the transformed plant. Bioinformatic analysis of the 5' and 3' flanking regions did not reveal disruption of known genes or creation of ORFs that would cause a safety issue. The stability of the inserted DNA was confirmed over several generations and a Mendelian inheritance pattern was demonstrated. The EFSA GMO Panel concluded that the molecular characterisation did not raise safety issues.

4. Comparative analysis

4.1. Evaluation of the relevant scientific data

The GMO Panel has considered the data on the compositional, agronomic and phenotypic characteristics of maize 3272 and its comparators as provided in the dossier and summarised below.

4.1.1. Choice of comparator and production of material for the compositional assessment

A comparative analysis of the compositional, agronomic and phenotypic characteristics of maize 3272 and its comparator was performed during field trials in the USA in six locations in 2003 and seven locations in 2004. The comparators used were negative segregants¹⁵ that had been isolated, after several stages of backcrossing and selfing of the progeny of the initial transformant, from maize that was essentially homozygous for the insert in maize 3272. The field trials were designed as randomised complete block designs with three replicates for each genotype.

The field trials performed in 2003 and 2004 were not considered by the EFSA GMO Panel as appropriate evidence for the absence of unintended effects owing to limitations in the study design, namely the use of a negative segregant as the only comparator. As negative segregants are derived from a GM organism, the GMO Panel does not consider them appropriate conventional counterparts with a history of safe use (EFSA, 2006).

¹² Additional information Oct 2012/Appendices A-2_01 and A-2_02.

¹³ Confidential information/Appendix 1.

¹⁴ Confidential information/Appendix 6.

¹⁵ Technical dossier/Section D7.2.

Following a request by the EFSA GMO Panel, the applicant provided additional compositional and agronomic data obtained from field trials where an appropriate conventional counterpart had been included.¹⁶ The new compositional analysis was performed on samples of 3272 maize, the corresponding non-GM comparator and a negative segregant grown at six locations in the USA in 2008. At each location, a randomised complete block design was used with three replicates per genotype. Agronomic and phenotypic data were also evaluated for 3272 maize, and its conventional counterpart in field trials performed at nine locations in the USA in 2008.

Data from field trials for the comparative assessment are necessary to identify potential unintended effects. Requirements for the geographical spread of locations and the number of growing seasons need to meet minimum standards of representativeness of environments and statistical power (EFSA, 2006). This affords confidence that any unintended effect will be detected if present. The field trials performed in 2008 did not fulfil the requirement for multiple seasons in the applicable EFSA Guidance Document (EFSA, 2006), nor did they fulfil the requirements specified in the current (EFSA, 2011a) guidance document. Because of this non-compliance with the requirements set out in its guidance, the EFSA GMO Panel considered the data provided as insufficient to exclude the possible presence of unintended effects.

4.2. Conclusion

The EFSA GMO Panel cannot conclude on the comparative assessment of the compositional, agronomic and phenotypic characteristics of maize 3272, on the basis of the data provided.

5. Food/feed safety assessment

5.1. Evaluation of relevant scientific data

In the absence of an appropriately performed comparative assessment, the EFSA GMO Panel is not in the position to conclude on the compositional characteristics of maize 3272 compared with conventional maize. The safety assessment, therefore, could not be completed and has focused mainly on the newly expressed proteins.

5.1.1. Effects of processing

Characteristics of the newly expressed proteins

The AMY797E protein (as contained in test substance AMY797E-0104) showed optimum enzymatic activity at ■■■ °C* and at pH ■■■.*

Phosphomannose isomerase as contained in test substance PMI-0105 showed optimum activity at pH 7.5. Substrate specificity of PMI has been further confirmed by a study in which various structurally similar saccharides were incubated with the microbially produced PMI-0105. Although PMI catalysed the interconversion between fructose-6-phosphate and mannose-6-phosphate at pH 7.5, no reaction occurred when other sugars or sugar phosphates were added as substrates.

The activity of a partially purified AMY797E protein from maize 3272 grain (AMY797E-0104) was determined by measuring the production of reducing sugars during the hydrolysis of potato starch. The effect of temperature on the AMY797E protein was also determined using an enzyme-linked immunosorbent assay (ELISA). After incubation for 30 minutes at temperatures from ■■■* to ■■■ °C,* there were slight changes in immunoreactivity in relation to the control sample (incubated at 4 °C), at ■■■ °C* the loss of immunoreactivity was ■■■%,* while at ■■■ °C* and higher temperatures immunoreactivity was lost completely.

¹⁶ Additional information January 2010.

* These data are withheld due to confidentiality claims by the applicant, pending the decision of the European Commission on confidentiality according to Article 30(2) of Regulation (EC) 1829/2003.

The influence of temperature on PMI derived from a recombinant *E. coli* strain (PMI-0198) was studied *in vitro* by determination of the specific activity after incubation of the enzyme at temperatures from 25 to 95 °C for 30 minutes. PMI activity was measured by the production of NADPH in a coupled spectrophotometric enzymatic activity assay. At 37 or 55 °C there was only a slight reduction when compared with the control (incubated at 25 °C), whereas at 65 and 95 °C the enzyme was almost completely inactivated. After incubation of the PMI protein (PMI-0105) at 4 to 37 °C for 30 minutes, there was no difference in enzyme activity, while the activity was lost completely at temperatures of 65 °C and higher. Using a specific ELISA the immunoreactivity after incubation at 25 and 37 °C in relation to the control sample (incubated at 4 °C) was practically the same while there was a loss of 95 % at 65 °C.

Newly expressed proteins in animal feed

The EFSA GMO Panel also requested information on the composition of by-products of the dry-grind ethanol process using maize 3272 compared with conventional maize, in particular in relation to the carbohydrate profile. For this purpose, the composition of dry distillers' grains with solubles (DDGS) produced from a mixture of ■ %* maize 3272 and ■ %* conventional maize was compared with that of DDGS produced from 100 % conventional maize to which microbially produced alpha-amylase was added (one sample each). The DDGS produced from both mixtures showed similar levels of protein, fat, fibre (crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF)), starch, total sugars, glucose and fructose.

The applicant, in response to a request from the GMO Panel, determined the activity of AMY797E alpha-amylase in wet distillers' grains (WDGs) derived from the dry-grind ethanol production process with a mixture of 96 % conventional maize and 4 % maize 3272. The alpha-amylase activity in all WDGs samples was below the limit of detection. Also AMY797E amylase could not be detected by ELISA. However, the results for WDGs spiked with AMY797E compared with the results for the purified enzyme showed a considerable decrease in the sensitivity of both assays when applied to WDGs, which raises doubts about the suitability of the detection methods.

Using a specific ELISA, the PMI protein was not detected (< 3 ng PMI/mL) in samples of WDGs obtained after processing of a mixture of grain sorghum (milo) and maize containing 4 % (v/v) grain from maize 3272.

5.1.2. Toxicology

5.1.2.1. Proteins used for safety assessment¹⁷

The AMY797E-containing test material used in the digestibility studies and the acute oral toxicity study (designated AMY797E-0104) was prepared from maize 3272 grain. The preparation had a specific alpha-amylase activity of about 33 000 U/g test substance. Using SDS-PAGE and protein staining as well as Western analysis, it was demonstrated that the AMY797E protein had the expected molecular weight (50.2 kDa). N-terminal sequence analysis showed that approximately 60 % of the AMY797E protein had the expected N-terminal sequence corresponding to the mature AMY797E protein, which is obtained after cleavage of the N-terminal maize gamma-zein peptide signal sequence (targeting the protein to the ER), while approximately 40 % corresponds to the mature protein with one additional amino acid removed. The test material used in the repeated-dose (28-day) oral toxicity study (designated AMY797E-0109) was also prepared from maize 3272 grain.

Given the low expression level of the PMI protein in maize 3272, recombinant proteins produced in *E. coli* (designated PMI-0198 or PMI-0105) were used for safety testing. The PMI in test substance PMI-0105 had the same amino acid sequence as the native *E. coli* protein (encoded by the *pml* (*manA*) gene). The PMI in test substance PMI-0198 contained an extension comprising 16 additional

¹⁷ Technical dossier/Section D7.8.1.

* These data are withheld due to confidentiality claims by the applicant, pending the decision of the European Commission on confidentiality according to Article 30(2) of Regulation (EC) 1829/2003.

amino acid residues at the N-terminus as a consequence of DNA sequence fusion used for production of the DNA encoding the recombinant protein. These 16 amino acids are encoded by additional DNA sequences derived from a T7 tag sequence (13 residues) and a polylinker sequence (three residues). PMI produced in maize 3272 was isolated from leaves and compared with the microbially produced PMI proteins through Western analysis and enzyme activity assay. Both the plant-expressed and the microbially produced PMI proteins showed an immunoreactive band in Western blots corresponding to the expected molecular weight (ca. 44.4 kDa for PMI-0198 and ca. 42.8 kDa for PMI-0105 and the plant expressed PMI protein), and were active in the enzyme activity assay.

5.1.2.2. Toxicological assessment of the newly expressed proteins in maize 3272

Alpha-amylases occur naturally in prokaryotes and eukaryotes including plants and animals used in food production, and thus are regularly consumed as part of the normal diet by humans and animals.

Phosphomannose isomerase enzymes have been purified from many organisms, including bacteria, yeast, rats, pigs and humans (Proudfoot et al., 1994), and have been demonstrated to be essential for many organisms, including *E. coli* (Markovitz et al., 1967) and fungi (Proudfoot et al., 1994). PMI activity is present in mammalian tissues, including skeletal muscle, brain, heart, liver, spleen, lung and placenta. The enzyme catalyses the conversion of mannose-6-phosphate to fructose-6-phosphate and vice versa, and these two compounds are the only known substrates of PMI enzymes (Freeze, 2002). The amino acid sequence of the PMI protein produced in maize 3272 (encoded by the *pmi* (*manA*) gene from *E. coli*) is identical to that of the PMI protein expressed in the GM maize MIR162, which has already been evaluated by the EFSA GMO Panel (EFSA, 2012).

(a) *Bioinformatic studies*

Bioinformatic analysis of the amino acid sequences of the AMY797E precursor protein and the PMI protein revealed no significant similarities to known toxic proteins.¹⁸

(b) *Resistance to degradation by proteolytic enzymes*

The resistance to degradation by pepsin of the AMY797E protein isolated from maize 3272 (AMY797E-0104) was studied in solutions at pH 1.2. The integrity of the test protein in samples taken at various time points was analysed. No intact protein (ca. 50.2 kDa) was seen within one minute of incubation using SDS PAGE followed by protein staining or Western analysis. Two new fragments were detected after this incubation period but they were not observed after five minutes.

The resistance to degradation by pepsin of the PMI protein isolated from a recombinant *E. coli* strain was studied in solutions at pH 1 to 1.2. The PMI protein was immediately degraded in the solutions containing pepsin at the initial concentration used.

In addition, the applicant provided a study where the resistance of PMI protein to pancreatin was studied in solutions at pH 7.5. The PMI protein was degraded within two minutes in solutions containing pancreatin at the initial concentration used. The EFSA GMO Panel notes that this study is not required by either the EFSA Guidance Document or Codex Alimentarius (CAC, 2009; EFSA, 2011a).

(c) *Acute toxicity testing*

The AMY797E protein (AMY797E-0104) and the PMI protein (PMI-0198) were tested for acute oral toxicity using mice in separate studies. No treatment-related adverse effects were observed after administration at a single dose of 1 500 mg AMY797E/kg bodyweight (bw) and 3 100 mg PMI/kg bw, respectively.

¹⁸ Technical dossier section D.7.8. and additional information received in September 2012.

The EFSA GMO Panel considers that acute toxicity testing of the newly expressed proteins is of little additional value for the risk assessment of the repeated human and animal consumption of food and feed derived from GM plants.

(d) *Repeated-dose toxicity testing*¹⁹

On request of the EFSA GMO Panel the applicant provided a repeated-dose 28-day toxicity study with the AMY797E protein using Wistar Han rats (CRL:WI(Han)). The study was performed according to OECD Guideline 407 and in compliance with the principles of good laboratory practice (GLP). The test material AMY797E-0109 containing 55.2 % (w/w) of the AMY797E protein was obtained from grain of maize 3272. Groups of five male and five female animals received the test material in aqueous solution by gavage at doses corresponding to 10, 55 or 550 mg AMY797E/kg bw per day for 28 consecutive days. One control group received the vehicle alone (0.5 % (w/w) carboxymethylcellulose (CMC) in water), and a second control group was administered bovine serum albumin (BSA) in the vehicle at a dose of 550 mg/kg bw per day (protein control group). It was shown that the AMY797E protein retained enzymatic activity in the vehicle for at least 28 hours at temperatures of 4 °C and ambient laboratory temperature (< 30 °C). In the statistical analysis the values obtained for the three test groups and the protein control group were compared with the vehicle control group.

One male animal in the protein control group was euthanised owing to its general poor condition; no cause of the clinical observations could be determined. Apart from this, regular observation and detailed examination of the animals revealed no notable clinical signs. There were no relevant differences neither in body weight development nor in food and water consumption between groups. There were no treatment-related ophthalmoscopy findings. Analysis of functional observation battery (FOB) parameters, including detailed clinical observations, quantitative functional observations and motor activity determination at the end of the treatment period showed a few statistically significant differences between the test groups and the vehicle control group, which are considered as isolated findings unrelated to treatment. The only significant difference in haematology, coagulation and clinical chemistry analyses was a higher mean cell haemoglobin concentration (MCHC) in females of the intermediate-dose group. In the absence of changes in other blood cell parameters, this difference, which was not observed in the high-dose group, is regarded as an incidental finding. Determination of the weights of selected organs and tissues at necropsy showed a significantly higher mean thymus weight for males of the high-dose group (absolute and after adjustment for terminal body weight). Since there were no histopathological findings in this organ and no changes in other parameters, indicating an effect on the immune system, the observed difference is not regarded as toxicologically relevant. Macroscopic and microscopic examinations of other organs and tissues revealed no notable differences in the incidence and severity of findings between groups. The EFSA GMO Panel concluded that administration of the AMY797E protein to rats for 28 days did not induce adverse effects up to the highest dose tested, i.e. 550 mg/kg bw per day.

5.1.2.3. Toxicological assessment of new constituents other than proteins and/or changed levels of natural constituents

No new constituents other than AMY797E and PMI were deliberately introduced and expressed in maize 3272. However, in the absence of an appropriately performed comparative assessment, the EFSA GMO Panel is not able to assess whether other unexpected constituents have been introduced or changed in concentration.

5.1.2.4. Animal studies with the food/feed derived from GM plants

(a) *Sub-chronic toxicity study*²⁰

¹⁹ Additional information 07/01/2010.

²⁰ Technical dossier section D.7.8.4 .

The applicant has provided a repeated-dose 90-day toxicity study in rats using grain of maize 3272 as a component of the diet. Groups of 12 male and 12 female Wistar-derived rats (Alpk:AP_{SD}) were fed diets containing 10 % or 41.5 % grain of maize 3272 for a period of 90 consecutive days. The control groups received diets containing 10 % or 41.5 % grain from a negative segregant (Syngenta maize amylase event 3272 negative isoline).

The EFSA GMO Panel considers that a repeated-dose 90-day oral toxicity study, in which material derived from a negative segregant is administered as the sole control material, is not adequate for the safety assessment of food/feed from GM plants. Therefore, the Panel did not consider this study in the evaluation.

(b) *Chicken feeding study*

A 49-day feeding study using broiler chickens was provided.²¹ A total of 900 Ross day-old broiler chicks were allocated to three groups, each group consisting of 300 broilers housed in 12 pens (25 birds per pen, six pens per sex). The three groups received diets containing maize 3272 (test group), a negative segregant (control group), or one non-GM commercial maize variety (reference group: NC2004). The EFSA GMO Panel considers that a study in which material derived from a negative segregant is administered as control material is not adequate for the safety assessment of food/feed from GM plants.

5.1.3. Allergenicity

The strategies used when assessing the potential allergenic risk focus on the characterisation of the source of the recombinant protein, the potential of the newly expressed protein to induce sensitisation or to elicit allergic reactions in already sensitised persons and whether the transformation may have altered the allergenic properties of the modified food.

5.1.3.1. Assessment of allergenicity of the newly expressed proteins²²

A weight-of-evidence approach is followed, taking into account all of the information obtained with various test methods, since no single experimental method yields decisive evidence for allergenicity (CAC, 2009; EFSA, 2006, 2011a).

The AMY797E protein in maize 3272 is an alpha-amylase protein which originates from alpha-amylase genes from three hyperthermophilic microorganisms of the order *Thermococcales*.

Bioinformatic analyses of the amino acid sequences of the AMY797E precursor protein using the criterion of 35 % identity in a window of 80 amino acids revealed no significant similarities to known allergens.

The applicant also performed an analysis searching for matches of eight contiguous identical amino acid sequences between the AMY797E precursor protein and known allergens. A match with the Per a 3 allergen from the American cockroach (*Periplaneta americana*) was identified. On request of the EFSA GMO Panel, the applicant performed a specific serum screening using five sera from individuals allergic to Per a 3. No immunoglobulin E (IgE) binding capacity of the five sera to the AMY797E protein was observed.

The studies described above provided information which did not indicate a likelihood of possible cross-reactivity between the AMY797E protein and known allergens.

In relation to the assessment of possible *de novo* sensitisation capacity of the AMY797E protein, the EFSA GMO Panel requested additional experimental data to the applicant because (i) some alpha-amylases are known to be allergens (see external report EFSA, 2009c); (ii) the newly expressed

²¹ Technical dossier/Section D.7.8.4/ Appendix 21.

²² Technical dossier/Section D7.9.1. and additional information received in January 2010 and September 2012.

AMY797E protein is an enzyme that is stable at high temperatures; and (iii) AMY797E originates from a source of which there is little information on its exposure to humans. The applicant presented additional information on a literature review of alpha-amylase studies relevant for the safety assessment; a review of enzyme allergy safety; expert views on the safety of the AMY797E protein; 3-D modelling data considered in this case as relevant for cross-reactivity assessment only; and an *in vitro* T-cell test where the AMY797E protein was not tested.²³ The EFSA GMO Panel is aware that for allergenicity assessment *in vitro* cell-based assays or *in vivo* tests on animal models have not yet been validated for regulatory purposes. However, the EFSA GMO Panel may consider them as additional information, e.g. on the potential for *de novo* sensitisation (EFSA, 2011a). Owing to the lack of bibliographic and/or experimental data on the absence of *de novo* sensitisation capacity specific to the AMY797E protein, the EFSA GMO Panel could not conclude on the potential for *de novo* allergic sensitisation of the newly expressed AMY797E protein.

The PMI protein in maize 3272 originates from *E. coli*. Bioinformatic analysis of the amino acid sequences of the PMI protein using the criterion of 35 % identity in a window of 80 amino acids revealed no significant similarities to known allergens. On request of the EFSA GMO Panel, the applicant provided additional information on the possible cross-reactivity of the PMI protein with the Hev b 13 latex allergen. Briefly, the applicant performed a bioinformatic analysis considering the introduction of sequence gaps for the calculation of the percentage identity as recommended by EFSA (EFSA, 2010a). A 28 % identity with the Hev b 13 protein was identified. This additional information was in line with previously assessed data on the PMI protein from maize MIR604 and MIR162 for which the EFSA GMO Panel issued opinions (EFSA, 2009a, 2012).

The applicant also performed a bioinformatic analysis searching for matches of eight contiguous identical amino acids between the PMI protein and known allergens. A match with the frog allergen α -parvalbumin was described. In line with application EFSA-GMO-UK-2005-11, the applicant provided a specific serum screening using serum from an allergic individual reported (Hilger et al., 2002) to react with the mentioned frog allergen, which was previously assessed by the EFSA GMO Panel (EFSA, 2009a).

The PMI is a member of the plant protein superfamily of cupins. Some members of this superfamily which have a specific 3-D structure are known to be complete (e.g. elicitor and sensitiser) allergens (Dunwell et al., 2001; Breiteneder and Radauer, 2004; Mills et al., 2004). In this context, allergenicity of PMI was previously assessed in maize MIR604 and MIR162 (EFSA, 2009a, 2012). On request of the EFSA GMO Panel, the applicant provided a study on 3-D modelling for PMI in maize 3272 that was in line with the data already assessed in maize MIR604. The study allowed the EFSA GMO Panel to conclude that there were no indications that the PMI protein may be allergenic (EFSA, 2009a).

The studies on resistance to degradation of the AMY797E and PMI proteins by proteolytic enzymes have been described in section 5.1.2.2.

Based on all the available information, the EFSA GMO Panel considers that there are no indications that the newly expressed PMI protein in maize 3272 may be allergenic. In relation to the AMY797E, the EFSA GMO Panel could not conclude on the *de novo* sensitisation potential of the protein.

5.1.3.2. Assessment of allergenicity of the whole GM plant or crop²⁴

According to the EFSA GMO Panel risk assessment guidelines (EFSA, 2006, 2011a), the applicant should test any potential change in the allergenicity of the whole GM plant by comparing the allergen repertoire with that of its appropriate comparator(s), when the plant receiving the introduced gene is known to be allergenic.

²³ Additional information received in September 2012.

²⁴ Technical dossier/Section 7.9.2.

Maize has not been considered to be a common allergenic food (EC, 2007). The prevalence of food allergy to maize is low and appears to vary with the geographic location (Moneret-Vautrin et al., 1998; Pastorello et al., 2009; Fonseca et al., 2012). At least 23 IgE-binding proteins have been identified in maize, a number of which are recognised as allergens. Sixteen of these proteins have been reported to be stress related, with LTP (lipid transfer protein) being the most important allergen (Pastorello et al., 2000, 2009; Pasini et al., 2002; Fonseca et al., 2012). In some studies, most individuals with a positive skin prick test (SPT) or having IgE antibodies against maize were suffering from a respiratory allergy and only a few displayed a true food allergy following oral challenge with maize products (Jones et al., 1995; Pasini et al., 2002). However, in another study of 27 patients with a claimed history of maize allergy one-half were found to be challenge-positive and thus had a food allergy to maize (Scibilia et al., 2008).

Since no reliable information is available from the comparative analysis and in light of its relevance for the identification of possible unintended effects, the EFSA GMO Panel cannot conclude on the allergenicity of the whole GM plant.

5.1.4. Nutritional assessment of the food/feed derived from GM plants

Maize grain 3272 is intended to serve as the source of amylase enzyme in the dry-grind ethanol process, and not to be used as a commodity crop. Therefore, the introduction of maize 3272 directly into the food and feed supply would be unintentional or accidental. However, by-products of processing maize 3272 will be extensively used as feed material.

The genetic modification in maize 3272 is not intended to alter nutritional parameters. The introduction of these products into the food and feed supply is therefore expected to have no nutritional impact, as compared with its conventional counterpart and non-GM maize varieties. However, in the absence of an appropriate compositional analysis this expectation could not be confirmed. Although no adverse effects were seen after feeding broilers with maize 3272, owing to the use of a negative segregant as control, the EFSA GMO Panel is not in the position to conclude on the nutritional assessment of maize 3272.

5.2. Conclusions

In the absence of an appropriately performed comparative assessment, the EFSA GMO Panel is not in the position to conclude on the compositional, agronomic and phenotypic characteristics of maize 3272, on the basis of the data provided. The safety assessment, therefore, could not be completed and has focused mainly on the newly expressed proteins AMY797E and PMI.

The AMY797E and PMI proteins did not show significant similarity to known toxins in bioinformatics analyses. The EFSA GMO Panel concluded that administration of the AMY797E protein to rats for 28 days did not induce adverse effects up to the highest dose tested. Based on all the available information, the EFSA GMO Panel considers that there are no indications that the newly expressed PMI protein in maize 3272 may be allergenic. In relation to the AMY797E, the EFSA GMO Panel could not conclude on the *de novo* sensitisation potential of the protein.

6. Environmental risk assessment and monitoring plan

6.1. Evaluation of relevant scientific data

The scope of this application, EFSA-GMO-UK-2006-34, is for food and feed uses, import and processing of maize 3272 and does not include cultivation. Considering the intended uses of maize 3272, the environmental risk assessment is concerned mainly through ingestion by animals, and their manure and faeces leading to exposure of gastrointestinal tract and soil microorganisms, and with the accidental release into the environment of viable maize 3272 grains (e.g. during transport and/or processing).

Maize 3272 has been developed to express a chimeric thermotolerant alpha-amylase (AMY797E) and a phosphomannose isomerase as a selectable marker (see section 3.1.2).

6.1.1. Environmental risk assessment

6.1.1.1. Potential unintended effects on plant fitness due to the genetic modification

Maize is highly domesticated and generally unable to survive in the environment without management intervention. Maize plants are not winter hardy in many regions of Europe; furthermore, they have lost their ability to release seeds from the cob and they do not occur outside cultivated land or disturbed habitats in agricultural landscapes of Europe, despite cultivation for many years. In cultivation, maize volunteers may arise under some environmental conditions (mild winters). Observations made on cobs, cob fragments or isolated grains shed in the field during harvesting indicate that grains may survive and overwinter in some regions, resulting in volunteers in subsequent crops. The occurrence of maize volunteers has been reported in Spain and other European regions (e.g. Gruber et al., 2008). However, maize volunteers have been shown to grow weakly and flower asynchronously with the maize crop (Palaudelmàs et al., 2009).

The applicant presented agronomic and phenotypic data gathered over a series of field trials conducted in the USA in 2003 and 2004. These field trials were not accepted by the EFSA GMO Panel owing to the use of a negative segregant as the only comparator. Upon request of the EFSA GMO Panel, the applicant provided additional data on agronomic and phenotypic characteristics of the GM maize from field studies in 2008. These field trials performed in 2008 did not fulfil the requirement for multiple seasons in the applicable EFSA Guidance Document (EFSA, 2006), nor did they fulfil the requirements specified in the current Guidance Document (EFSA, 2011a) (for further details, please see section 4.1.1).

However, in accordance with its Guidance Document on the environmental risk assessment of GM plants (EFSA, 2010b), the EFSA GMO Panel follows a weight of evidence approach in collating and assessing appropriate information from various data sources (e.g. molecular and compositional data, available agronomic and phenotypic data from field trials performed by the applicant, literature) in order to assess the likelihood of unintended effects on the environment. The applicant provided molecular and compositional data that are assessed by the EFSA GMO Panel in sections 3 and 4, respectively. In addition, the applicant provided data on the most relevant phenotypic (e.g. crop physiology, morphology, development) and agronomic (e.g. yield, percentage of emerged plants, plant population at harvest) characteristics of maize 3272, in order to assess the agronomic performance of the GM maize.

In addition to the data presented by the applicant, the EFSA GMO Panel is not aware of any scientific report of increased spread and establishment of maize 3272 or maize with comparable properties, or of any change in survival capacity, including overwintering. In addition, the ability to utilise mannose can be regarded as selective advantage only where and when mannose is available as a carbon source, which is not the case in soils.

Therefore, given the limited field trial data provided by the applicant to support its risk assessment of maize 3272, the EFSA GMO Panel has identified a gap in the data on the agronomic and phenotypic characterisation of this event and considers that uncertainty over the data remains. However, the EFSA GMO Panel concludes that, considering the scope of this application, the weight of evidence approach from different sources of available data and the poor ability of maize to survive outside cultivated land, there is very little likelihood that maize 3272 has any enhanced fitness characteristics that will change its persistence and survival following accidental release into the environment of viable grains from maize 3272.

6.1.1.2. Potential for gene transfer²⁵

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either horizontal gene transfer of DNA or vertical gene transfer via seed spillage followed by cross-pollination.

(a) *Plant to bacteria gene transfer*

Genomic plant DNA is a component of several food and feed products derived from maize. It is well documented that DNA present in food and feed becomes substantially degraded during processing and digestion in the human or animal gastrointestinal tract. However, a low level of exposure of fragments of ingested DNA, including the recombinant fraction of such DNA, to bacteria and other microorganisms in the digestive tract of humans, domesticated animals and other environments exposed to the GM plant or plant material is expected.

Current scientific knowledge of recombination processes in bacteria suggests that horizontal transfer of non-mobile, chromosomally located DNA fragments between unrelated organisms (such as plants to bacteria) is not likely occur at detectable frequencies under natural conditions (see EFSA, 2009b, for further details).

A successful horizontal transfer would require stable insertion of the transgene sequences into a bacterial genome and a selective advantage to be conferred on the transformed host. The only mechanism known to facilitate horizontal transfer of non-mobile, chromosomal DNA fragments to microbial genomes is homologous recombination. This requires the presence of stretches of DNA sequences that are similar in the recombining DNA molecules and, in addition to substitutive gene replacement, facilitates the insertion of non-homologous DNA sequences if their flanking regions share sequence similarity with bacterial sequences in the recipient.

Maize 3272 was developed through *A. tumefaciens*-mediated transformation and contains two recombinant genetic elements which consist of DNA sequences with high similarity to those of prokaryotes (*Bacteria*, *Archaea*). One element contains the modified *amy797E* gene, a synthetic gene, encoding for a chimeric thermotolerant alpha-amylase, made of three gene segments, two of which are derived from sequences of *Thermococcus* sp. strains and one which is isolated from an enrichment culture of extreme thermophilic microorganisms (90 °C) from marine hydrothermal systems. Based on DNA sequence similarity, the latter gene sequence was found to be closely related to *Thermococcus* or *Pyrococcus*. Both *Thermococcus* and *Pyrococcus* belong to the domain *Archaea*, and thus are not bacteria. The other genetic element contains the *pmi* gene, encoding a phosphomannose isomerase, and is derived from *E. coli*. The genetic sequence encoding for the alpha-amylase has been codon-optimised for expression in plants. Promoters for both genetic elements originate from maize (for further details, see section 3). Terminator regions of the first genetic element come from the 35S gene of the *Cauliflower mosaic virus* and of the second from *A. tumefaciens* (*nos* gene).

Molecular analyses indicated sequence similarity to DNA of the Ti plasmid of *A. tumefaciens* both at the 5' and 3' ends of the insert corresponding to a length of 143 bp and 253 bp, respectively. Therefore, there is a possibility of double homologous recombination resulting in a replacement of the *nos* gene in the Ti plasmid of *A. tumefaciens* by the insert containing the alpha-amylase and *pmi* cassettes. The deletion of the *nos* gene should cause a selective disadvantage for *A. tumefaciens* as the tumour induction in plants would be impaired. Further dissemination of the newly acquired alpha-amylase and *pmi* in the Ti plasmid to bacteria would be limited to the relatives of *Agrobacterium* within the Rhizobiaceae owing to the host range specificity of the Ti plasmid (Holsters et al., 1978; Cook et al., 1997; Teyssier-Cuvelle et al., 1999). No selective advantage is expected for bacteria expressing a thermotolerant alpha-amylase. Furthermore, bacteria with a capacity to utilise mannose as a carbon source are common in soil.

²⁵ Technical dossier/section D6 and D9.3.

While certain members of the domain *Archaea* can be found as regular inhabitants of the gastrointestinal tract of humans and animals, the presence of members of the genus *Thermococcus* or *Pyrococcus*, which are adapted to extremely high temperatures, is not expected. Natural variants of other alpha-amylases can occur in different bacteria, e.g. members of the lactobacilli and spore-forming Firmicutes (including the *Bacillus* group), which can be among the natural microbial gut inhabitants. Homologous recombination of the sequence encoding for the chimeric thermotolerant alpha-amylase, derived from *Archaea*, with natural variants of such genes is highly unlikely because of the lack of homologous sequences in the main receiving environments, i.e. the gut of humans or animals. It cannot be excluded that sequences with similarity to the *pmi* genes of *E. coli* are present in bacteria residing in the gastrointestinal tract of humans or animals. Homologous recombination between *pmi* of maize 3272 and natural variants of these genes resulting in horizontal gene transfer would, however, only replace natural variants (i.e. substitutive recombination) and are therefore unlikely to confer a new trait to the recipient organisms (EFSA, 2009b). The promoter, which originates from maize and is expected to be less functional in bacteria (Warren et al., 2008), makes it unlikely that a replacement of a natural variant of the *pmi* gene in environmental bacteria by the *pmi* gene of maize 3272 would confer a selective advantage.

In addition to homology-based recombination processes, illegitimate recombination that does not require similarity between the recombining DNA molecules is theoretically possible. However, transformation rates for illegitimate recombination are considered to be 10^{10} -fold lower than for homologous recombination (Hülter and Wackernagel, 2008; EFSA, 2009b). Illegitimate recombination events have not been detected in studies that have exposed bacteria to high concentrations of GM-plant DNA (EFSA, 2009b). Thus, this process, compared with homologous recombination, is considered not to contribute significantly to horizontal gene transfer events. In comparison with the above-described homology-facilitated recombination processes, the contribution of illegitimate recombination is extremely low.

Owing to the natural occurrence of alpha-amylase encoding genes and *pmi* genes in the environment, a low-level gene transfer to natural prokaryotic recipients is thought not to confer a new trait and selective advantage. Considering its intended uses as food and feed and the above assessment, the EFSA GMO Panel has therefore not identified a concern associated with a horizontal gene transfer from maize 3272 to prokaryotes (i.e. bacteria, *Archaea*).

(b) *Plant-to-plant gene transfer*

Considering the intended uses of maize 3272 and the physical characteristics of maize grains, possible pathways of gene dispersal are grain spillage and the dispersal of pollen from occasional feral GM maize plants originating from accidental grain spillage during transport and/or processing.

The extent of cross-pollination to other maize varieties will mainly depend on the scale of accidental release during transport and processing and on successful establishment and subsequent flowering of this GM maize plant. For maize, any vertical gene transfer is limited to other *Zea mays* plants as populations of sexually compatible wild relatives of maize are not known in Europe (Eastham and Sweet, 2002; OECD, 2003).

The flowering of occasional feral GM maize plants originating from accidental release during transport and processing is unlikely to disperse significant amounts of GM maize pollen to other maize plants. Field observations performed on maize volunteers after GM maize cultivation in Spain revealed that maize volunteers had a low vigour, rarely had cobs and produced pollen that cross-pollinated neighbour plants only at low levels (Palaudelmàs et al., 2009).

Although GM maize plants outside cropped area have been reported in Korea, as a result of grain spillage during import, transport, storage, handling and processing (Kim et al., 2006; Lee et al., 2009; Park et al., 2010), survival of maize plants outside cultivation in Europe is mainly limited by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and frost. As for any other maize varieties, GM maize plants would survive in

subsequent seasons only in warmer regions of Europe and are not likely to establish feral populations under European environmental conditions.

The EFSA GMO Panel takes into account that this application does not include cultivation of maize 3272 within the EU so that the likelihood of cross-pollination between cultivated maize and the occasional feral maize plants resulting from grain spillage is considered extremely low. In conclusion, considering the scope of this application, a weight of evidence approach from different sources of available data and the poor ability of maize to survive outside cultivated land, the EFSA GMO Panel is of the opinion that there is very little likelihood of adverse environmental effects as a consequence of spread of genes from this GM maize in Europe.

6.1.1.3. Potential interactions of the GM plant with target organisms

Considering the intended uses of maize 3272, excluding cultivation, and the absence of target organisms, potential interactions of the GM plant with target organisms were not considered an issue by the EFSA GMO Panel.

6.1.1.4. Potential interactions of the GM plant with non-target organisms

Owing to the intended uses of maize 3272, which exclude cultivation, and the low level of exposure to the environment, potential interactions of the GM plant with non-target organisms were not considered an issue by the EFSA GMO Panel.

6.1.1.5. Potential interaction with the abiotic environment and biogeochemical cycles

Owing to the intended uses of maize 3272, which exclude cultivation, and the low level of exposure to the environment, potential interactions with the abiotic environment and biogeochemical cycles were not considered an issue by the EFSA GMO Panel.

6.2. Post-market environmental monitoring

The objectives of a post-market environmental monitoring (PMEM) plan according to Annex VII of Directive 2001/18/EC are (1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the environmental risk assessment (ERA) are correct; and (2) to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment that were not anticipated in the ERA.

Monitoring is related to risk management, and thus a final adoption of the PMEM plan falls outside the mandate of EFSA. However, the EFSA GMO Panel gives its opinion on the scientific content of the PMEM plan provided by the applicant (EFSA, 2011b). The potential exposure to the environment, including humans and animals, of maize 3272 would be mainly through ingestion by animals, and their manure and faeces leading to exposure of gastrointestinal tract and soil microorganisms, and with the accidental release into the environment of viable maize 3272 grains during transport and/or processing.

The scope of the PMEM plan provided by the applicant is in line with the intended uses. As the ERA did not identify potential adverse environmental effects due to maize 3272, no case-specific monitoring is required.

The PMEM plan proposed by the applicant includes (1) the description of an approach involving operators (federations involved in maize import and processing), reporting to the applicant, via a centralised system, any observed adverse effect(s) of GMOs on human health and the environment; (2) a coordinating system established by EuropaBio for the collection of the information recorded by the various operators; and (3) the use of networks of existing surveillance systems (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis.

The EFSA GMO Panel is of the opinion that the scope of the PMEM plan proposed by the applicant is in line with the intended uses of maize 3272 as the ERA did not cover cultivation and identified no

potential adverse environmental effects. The EFSA GMO Panel agrees with the reporting intervals proposed by the applicant in the PMEM plan.

6.3. Conclusion

The scope of the application includes food and feed uses, import and processing of maize 3272 and excludes cultivation. Considering the intended uses of maize 3272, the ERA is concerned with indirect exposure, mainly through ingestion by animals, and their manure and faeces leading to exposure of gastrointestinal tract and soil microorganisms, and with the accidental release into the environment of viable maize 3272 grains during transport and/or processing. The EFSA GMO Panel has identified a gap in the data on the agronomic and phenotypic characterisation of GM maize 3272 and considers that uncertainty over these characteristics remains. However, considering the scope of this application, a weight of evidence approach from different sources of available data and the poor ability of maize to survive outside cultivated land, the EFSA GMO Panel concluded that there is very little likelihood of any adverse environmental impacts due to the accidental release into the environment of viable grains from maize 3272. Considering its intended uses as food and feed, interactions with the biotic and abiotic environment were not considered to be an issue. Risks associated with a theoretically possible horizontal gene transfer from maize 3272 to prokaryotes (i.e. bacteria, *Archaea*) have been analysed and did not raise safety concerns. The scope of the PMEM plan provided by the applicant and the reporting intervals were in line with the intended uses of maize 3272 and the guidance document of the EFSA GMO Panel on PMEM of GM plants (EFSA, 2011b). The EFSA GMO Panel agreed with the reporting intervals proposed by the applicant in the PMEM plan.

CONCLUSIONS AND RECOMMENDATIONS

The molecular characterisation data established that the GM maize 3272 contains a single insert consisting of the *amy797E* and the *pmi* cassettes. Bioinformatic analyses and genetic stability studies did not raise safety issues. The levels of the AMY797E and PMI protein in maize 3272 have been sufficiently analysed.

In the absence of an appropriately performed comparative assessment, the EFSA GMO Panel is not in the position to conclude on the compositional, agronomic and phenotypic characteristics of maize 3272, on the basis of the data provided. The safety assessment could therefore not be completed, and has focused mainly on the newly expressed proteins AMY797E and PMI. The AMY797E and PMI proteins did not show significant similarity to known toxins in bioinformatic analyses. The EFSA GMO Panel concluded that administration of the AMY797E protein to rats for 28 days did not induce adverse effects up to the highest dose tested. Based on all the available information, the EFSA GMO Panel considers that there are no indications that the newly expressed PMI protein in maize 3272 may be allergenic. In relation to the AMY797E protein, the EFSA GMO Panel could not conclude on the *de novo* sensitisation potential of the protein.

Considering the intended uses of maize 3272, the ERA is concerned with indirect exposure, mainly through ingestion by animals, and their manure and faeces leading to exposure of gastrointestinal tract and soil microorganisms, and with the accidental release into the environment of viable maize 3272 grains during transport and/or processing. The EFSA GMO Panel has identified a gap in the data on the agronomic and phenotypic characterisation of GM maize 3272 and considers that uncertainty over these characteristics remains. However, considering the scope of this application, a weight of evidence approach from different sources of available data and the poor ability of maize to survive outside cultivated land, the EFSA GMO Panel concluded that there is very little likelihood of any adverse environmental impacts due to the accidental release into the environment of viable grains from maize 3272. Considering its intended uses as food and feed, interactions with the biotic and abiotic environment were not considered to be an issue. Risks associated with a theoretically possible horizontal gene transfer from maize 3272 to prokaryotes (i.e. bacteria, *Archaea*) have been analysed and did not raise safety concerns. The scope of the PMEM plan provided by the applicant and the reporting intervals were in line with the intended uses of maize 3272 and the guidance document of

the EFSA GMO Panel on PMEM of GM plants (EFSA, 2011b). The EFSA GMO Panel agreed with the reporting intervals proposed by the applicant in the PMEM plan.

In the absence of an appropriately performed comparative assessment by the applicant, the EFSA GMO Panel was not in a position to complete its risk assessment on maize 3272 and therefore did not conclude on the safety of maize 3272 compared with its conventional counterpart with respect to potential effects on human and animal health. However, the EFSA GMO Panel concluded that the maize event 3272 is unlikely to have any adverse effect on the environment in the context of its intended uses.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from the Competent Authority of the United Kingdom, received on 9 March 2006, concerning a request for the placing on the market of genetically modified maize 3272 submitted under Regulation (EC) No 1829/2003 by Syngenta Crop Protection AG.
2. Acknowledgement letter, dated 16 March 2006, from EFSA to the Competent Authority of the United Kingdom.
3. Letter from EFSA to applicant, dated 26 March 2007, requesting additional information under completeness check.
4. Letter from applicant to EFSA, received on 4 June 2007, providing additional information under completeness check.
5. Letter from EFSA to applicant, dated 6 July 2007, delivering the “Statement of Validity” for application EFSA-GMO-UK-2006-34, regarding genetically modified maize 3272 submitted under Regulation (EC) No 1829/2003 by Syngenta Crop Protection AG.
6. Letter from EFSA/JRC to applicant, dated 11 July 2007, requesting additional information (JRC) and stopping the clock.
7. Letter from EFSA to applicant, dated 23 November 2007, requesting additional information and maintaining the clock stopped.
8. Letter from EFSA to applicant, dated 6 December 2007, re-starting the clock for the JRC and maintaining the clock stopped for EFSA.
9. Letter from EFSA to applicant, dated 14 April 2008, requesting additional information and maintaining the clock stopped.
10. Letter from applicant to EFSA, received on 7 January 2010, providing additional information.
11. Letter from EFSA to applicant, dated 6 May 2010, requesting additional information and maintaining the clock stopped.
12. Letter from applicant to EFSA, received on 1 July 2010, requesting clarifications on EFSA’s request for additional information.
13. Letter from EFSA to applicant, dated 26 July 2010, providing clarifications on additional information.
14. Letter from EFSA to applicant, dated 11 July 2012, informing on finalisation of risk assessment with available information.
15. Letter from applicant to EFSA, received on 1 October 2012, providing additional information.

16. Letter from EFSA to applicant, dated 28 May 2013, re-starting the clock.

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